



Capturing nucleic acid variants with precision using CRISPR diagnostics

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ABSTRACT

CRISPR/Cas systems have the ability to precisely target nucleotide sequences and enable their rapid identification and modification. While nucleotide modification has enabled the therapeutic correction of diseases, the process of identifying the target DNA or RNA has greatly expanded the field of molecular diagnostics in recent times. CRISPR-based DNA/RNA detection through programmable nucleic acid binding or cleavage has been demonstrated for a large number of pathogenic and non-pathogenic targets. Combining CRISPR detection with nucleic acid amplification and a terminal signal readout step allowed the development of numerous rapid and robust nucleic acid platforms. Wherever the Cas effector can faithfully distinguish nucleobase variants in the target, the platform can also be extended for sequencing-free rapid variant detection. Some initial PAM disruption-based SNV detection reports were limited to finding or integrating mutated/mismatched nucleotides within the PAM sequences. In this review, we try to summarize the developments made in CRISPR diagnostics (CRISPRDx) to date emphasizing CRISPR-based SNV detection. We also discuss the applications where such diagnostic modalities can be put to use, covering various fields of clinical research, SNV screens, disease genotyping, primary surveillance during microbial infections, agriculture, food safety, and industrial biotechnology. The ease of rapid design and implementation of such multiplexable assays can potentially expand the applications of CRISPRDx in the domain of affinity-based target sequencing, with immense possibilities for low-cost, quick, and widespread usage. In the end, in combination with proximity assays and a suicidal gene approach, CRISPR-based *in vivo* SNV detection and cancer cell targeting can be formulated as personalized gene therapy.

1. Introduction

Alterations depending on their position in the genome can either be beneficial or detrimental subjecting to gene context, expression status, and disease state. Single nucleotide variants (SNVs), collectively representing a category of nucleotide changes at a specific locus in the genome, often constitute hallmarks of disease states and are important for disease stratification and diagnosis (Cargill et al., 1999; Syvänen, 2001; Sachidanandam et al., 2001; Lazarus et al., 2002; Katsonis et al., 2014; Deng et al., 2017). This change in a nucleotide sequence can remain unnoticed due to codon degeneracy (synonymous variant) or can lead to a change in the amino acid (non-synonymous variant) sequence. Further, depending on the effect of the amino acid substitution on protein structure and function, a variant can augment or diminish its role. It can even result in certain SNVs being pathogenic. Sometimes even a single SNP in humans can cause certain Mendelian diseases, or sometimes a combination of such aberrations can manifest as complex

diseases. Notably, SNVs can also perturb other disease-causing agents like bacteria or viruses by making them competitively fitter or weaker. Therefore, early detection or prognosis of SNVs can be valuable in understanding the risk and management of the disease. SNVs can be assayed using strategically designed hybridization techniques or sequencing methods. However, most sequencing methods take time, use multiple resources, and are generally unsuitable for point-of-care applications. Thus low-cost, rapid, and robust methods independent of sequencing can significantly augment variant identification across samples, particularly in resource-limited settings (Chiu and Miller, 2019; Vandenberg et al., 2021).

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins or Cas effectors are RNA-guided programmable nucleases that can specifically identify and modify nucleic acids. Wherein typically a short-length spacer RNA (~20–28 nucleotides) sequence complementary to target DNA or RNA sequences is used to guide the RNP complex to perform Cas-effector-dependent

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enzymatic activity. CRISPR-Cas systems identified so far can be divided into two classes. Whereas Class 1 systems employ a complex of multiple RNA-guided Cas proteins to degrade target nucleic acids, Class 2, uses a single large RNA-guided Cas effector for the DNA cleavage activity. Further, Class 1 is partitioned into types I, III, and IV, and class 2 into types II, V, and VI. These six types are divided again into 19 subtypes by exclusively categorizing Cas effectors for their activities (Makarova et al., 2011, 2020). Among these much-explored canonical CRISPR-Cas9 systems utilizes an RNA-guided Cas9 endonuclease to create double-stranded breaks at a target DNA. But unlike Cas9 some of the later discovered and much studied Cas effectors like Cas13, Cas12 and Cas14 show an alternative trans-cleavage or collateral activity, which is activated through the complementary base-pairing of the guide-RNA and an activator target RNA/ssDNA or dsDNA sequence to nonspecifically cleave nearby RNA and ssDNA (single-stranded DNA), respectively.

The CRISPR/Cas systems have moved beyond being just a primary gene-editing tool to a platform with multi-faceted aptitudes (Martin et al., 2012; Ran et al., 2013; Wang et al., 2016; Adli, 2018). Attachment of Cas proteins with different DNA/RNA effectors and modulators has expanded the toolset to avenues such as gene expression regulation, *in situ* labeling, and base editing (Larson et al., 2013; Gilbert et al., 2014; Konermann et al., 2015; Deng et al., 2015; Rees and Liu, 2018; Anzalone et al., 2020). During this course of finding the best effector, a range of Cas proteins have also been shown to discriminate sequences with even a single nucleotide change (Wu et al., 2014; Myhrvold et al., 2018; Harrington et al., 2018; Teng et al., 2019; Acharya et al., 2019; Azhar et al., 2021).

Interestingly, different CRISPR proteins capable of discriminating SNVs can be used for developing rapid and deployable assays for detecting single nucleotide changes, eliminating the use of laborious and expensive sequencing-based methods. The cornerstones of any effective diagnostic regimen are high sensitivity and specificity. While the specificity of CRISPR systems has improved over the last few years, the conversion of a biological process of DNA recognition to a robust downstream readout has necessitated innovations at the level of signal sensitivity. This has ranged from signal improvement at the source (input material) through amplification of nucleic acids to the development of more sensitive detectors culminating in handheld or wearable devices (Wang et al., 2020; Kaminski et al., 2021; Nguyen et al., 2021). During the recent COVID-19 pandemic several of these diagnostic techniques have documented the applicability of CRISPR diagnostics (CRISPRDx) for identifying CoV-2 signatures in patient samples (Broughton et al., 2020; Joung et al., 2020; Guo et al., 2020; Ding et al., 2020; Wang et al., 2021a; Fozouni et al., 2021; Azhar et al., 2021). However, as the virus is rapidly mutating into different clades, rapid identification and continuous monitoring is the only way for its containment thereby necessitating the continuous development of variant detection strategies that are publicly deployable (Kumar et al., 2021; de Puig et al., 2021; Wang et al., 2021a).

Cas effectors can show single-base specificity though only a handful of these possesses SNV/ SNP detection abilities through spacer sequence mismatch sensitivity, such as Cas13a, Cas12a, Cas12b, Cas14a, and Cas9, etc. (Myhrvold et al., 2018; Li et al., 2018; Teng et al., 2019; Harrington et al., 2018; Balderston et al., 2021). Among Cas9 proteins, the Cas9 ortholog from *Francisella novicida* (FnCas9) has also been reported to discriminate target sequences having a single nucleotide change, with its low affinity towards mismatched targets (Acharya et al., 2019; Azhar et al., 2021; Kumar et al., 2021; Chakraborty et al., 2021; Gulati et al., 2021). In the following review, we primarily focus on the ability of different Cas systems for nucleobase discrimination and its applications to develop variant screening assays.

2. Cas systems for nucleic acid detection

An ideal nucleic acid detection platform strives to achieve point-of-care diagnostics that are inexpensive, rapid, sensitive, specific, and

robust. Many of the initial methodologies for such detection systems were built either around polymerase chain reaction (PCR) or isothermal amplification-based probe detection. It was soon realized that direct probe-based detection can often result in non-specific readouts. Thus, adding a second level of accuracy through specific detection of byproducts developed from the amplification reaction can greatly improve the degree of specificity. For this reason, the single-nucleotide specificity of certain CRISPR/Cas systems makes them an ideal fit when either used alone or in combination for developing highly specific nucleic acid detection platforms that can cater to multiple applications, Fig. 1.

CRISPR diagnostics or CRISPRDx encompasses methodologies for both polynucleotide detection and identifying nucleobase identities. The CRISPR effector-based nucleic acid detection was first described with the Cas9-based ZIKA virus diagnostic platform (Pardee et al., 2016). This inspired studies to discover previously unexplored Cas effectors such as Cas13, Cas12, and Cas14 for developing rapid nucleic acid detection assays, Fig. 1 (Myhrvold et al., 2018; Li et al., 2018; Teng et al., 2019; Harrington et al., 2018). Nearly all of these proteins work through gRNA-based activation upon recognizing a target sequence and subsequent signal amplification through their ability to perform trans-cleavage activity on nearby fluorescently labeled nucleic acid probes. Hence, recognition of a complementary activator sequence by gRNA: Cas RNP complex leads to concomitant cleavage of polynucleotide sequences present in the vicinity. For example, Cas13a shows trans-cleavage after encountering target RNA substrate molecules and begins to indiscriminately chop any RNA molecules nearby upon activation. Similarly, Cas12 once activated with gRNA complementary activator ssDNA/dsDNA sequences, can perform nonspecific cleavage of any ssDNA present locally. Lately, nucleic acid detection assays focused on multi-subunit complex type I and type III CRISPR systems have also been explored (Yoshimi et al., 2020; Santiago-Frangos et al., 2021).

One of the first CRISPR-based nucleic acid detection assays was called NASBACC (Nucleic Acid Sequence-Based Amplification CRISPR Cleavage). In this method, detection happens through the ability of Cas9 protein to selectively cleave target DNA, in combination with toehold switch sensors. NASBACC, a portable, sensitive, and low-cost nucleic acid diagnostic platform for ZIKA virus detection, was also shown to adapt to programmable sensor workflow for high throughput and rapid responses. Moreover, this assay could also be used to discriminate between different ZIKA strains in which nucleotide variants can lie within the protospacer-adjacent motif (PAM) and disrupt the Cas9 binding activity (Pardee et al., 2016).

With the discovery of Cas13a that could target RNA, the field of CRISPR diagnostics accelerated the detection of RNA sequences through a reporter-based modality. This output was generated by trans-cleavage of ssRNA sequences upon Cas13a recognizing its bona fide target. The novel RNA targeting and cleavage ability of Cas13a led to the development of another nucleic acid detection platform termed SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing, Fig. 1 (Jonathan S Gootenberg et al., 2017)). SHERLOCK can be rapid, sensitive, and adaptable to point-of-care pathogen detection, genotyping, and disease monitoring. Once in conjunction with an isothermal amplification step and Cas13a-based mismatch sensitivity, SHERLOCK can detect and discriminate between different strains of Zika and Dengue virus (Jonathan S Gootenberg et al., 2017).

Similar to Cas13a, DNA-targeting CRISPR effectors with similar functionality also exists. Cas12a (Shmakov et al., 2015; Chen et al., 2018; Swarts and Jinek, 2019) and Cas14a (Harrington et al., 2018) with their trans-cleavage of ssDNA reporters, allowed the expansion of DNA targeting CRISPRDx to multiple applications. In particular, Cas12a, due to its gRNA-programmed specific target detection and activation of direct collateral cleavage of complementary single-stranded DNA targets used as short readout probes have been widely used for the identification of pathogenic sequences with high precision. Since it combines high target specificity and has the advantage of handling stable DNA probes

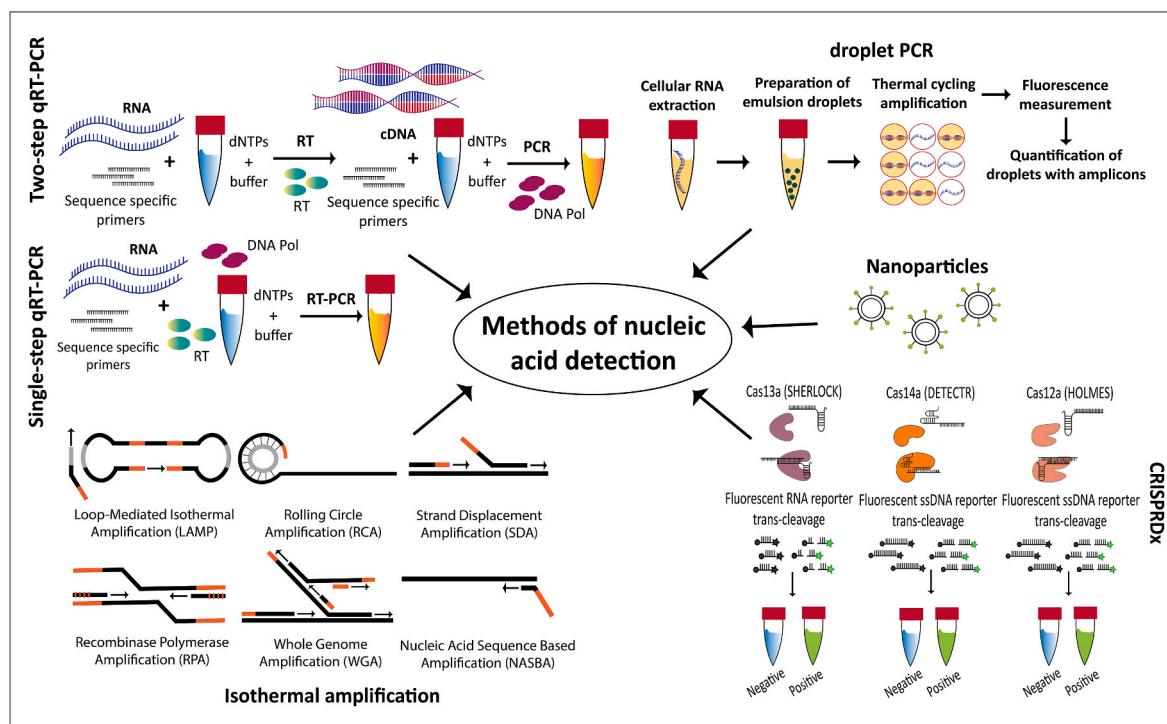


Fig. 1. Schematic showing the various methods used for nucleic acid detection to develop point-of-care diagnostics such as Two-step or single-step quantitative RT-PCR-based, droplet-PCR-based, isothermal amplification-based, nanoparticle-based, and much recent CRISPR/Cas-based.

(compared to RNA probes), Cas12a has been used in more detection assays than other Cas effectors (Wang et al., 2020; van Dongen et al., 2020; Aman et al., 2020; Wang et al., 2020).

Recently, the type III CRISPR systems activated through gRNA triggered sequence-specific recognition of target RNA by CRISPR-Csm complex, have also been reported for nucleic acid detection. Once activated, Cas10 carries out nucleotide polymerization and uses ATP to make cyclic-oligonucleotides, pyrophosphates, and protons, which are then released as byproducts to give detectable readouts (Santiago-Frangos et al., 2021). Unlike type III CRISPR systems, type I CRISPR systems-based assays work very similar to Cas12a effectors, by gRNA programmed target dsDNA detection and activated complementary single-stranded DNA targets trans-cleavage (Yoshimi et al., 2020).

Although CRISPR-based molecular diagnostic assays are quick and robust, it is crucial to understand the importance of three key steps involved: 1) direct amplification (when starting material is DNA) or reverse transcription (RT)-amplification (when starting material is

RNA), 2) treatment with a Cas effector, and 3) signal readout from the assay. Since different Cas effectors can show cleavage or collateral-cleavage activities depending on their target-induced activation, different amplification methods and post-detection signal readouts contribute to increasing the sensitivity and scalability of their applications, Fig. 2, which are discussed in detail in the next section.

2.1. Different amplification techniques are used along with CRISPR-based diagnostics

Amplification methods are required to get a detectable signal readout through CRISPR-based molecular diagnostic assays, particularly for low-copy targets. Different Cas proteins, depending on their respective structures, show varying levels of sensitivity to reaction temperatures. This in itself can be a bottleneck while selecting an amplification method as depending on the individual Cas protein's active temperature range this step can be done along with the Cas/

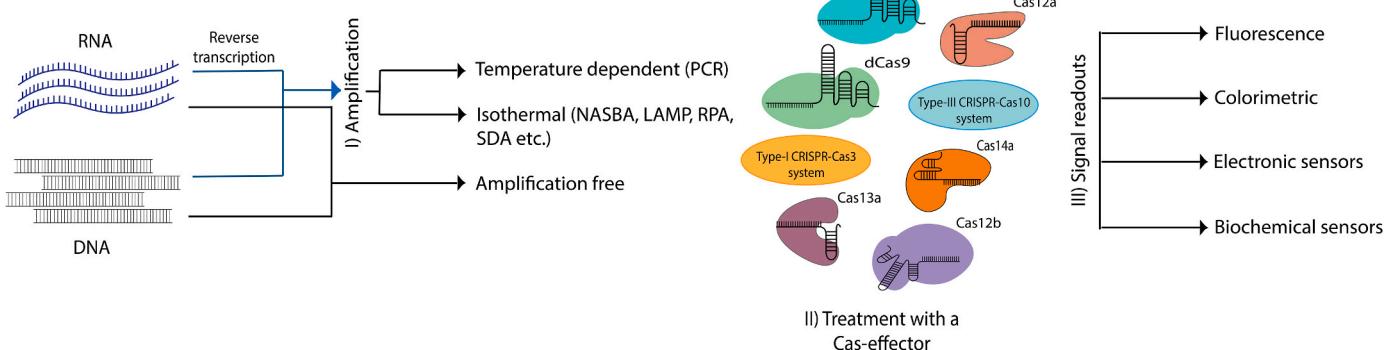


Fig. 2. Choice of different nucleic acid amplification techniques and signal-readouts used along with CRISPR-based diagnostics. Three key steps involved CRISPR-based molecular diagnostic assays: I) direct amplification (when starting material is DNA) or reverse transcription (RT) -amplification (when the starting material is RNA), II) treatment with a Cas effector and III) signal readout.

crRNA effector as a single reaction or separately as an independent pre-CRISPR reaction. Eventually, these can be categorized into temperature-dependent or isothermal amplification methods.

DNA amplification is relatively straightforward, through multiple adaptations of a primer pair-initiated thermal cycling process called polymerase chain reaction (PCR). This process, however, requires a thermal cycler machine, which might be difficult to arrange in resource-limited field settings (van Dongen et al., 2020). On the other hand, cyclic temperature changes during PCR limit the development of single reaction tubes running both amplification and CRISPR reactions, an important consideration while making contamination-free, universally adaptable diagnostic procedures with minimum liquid handling. These limitations can be majorly overcome through developments made in the field of isothermal amplification such as Loop-Mediated Isothermal Amplification (LAMP) or Recombinase Polymerase Amplification (RPA). By combining these with CRISPR reaction, an integrated diagnostic assay performed at a single temperature becomes feasible, reducing cross-contamination or multi-step handling errors.

Single-pot reactions have to take into account the effect of all the components on reaction efficiency. Recombinase polymerase amplification or recombinase-aided amplification (RPA/RAA) are popular methods for isothermal amplification and are usually performed at a temperature range of 37–42 °C (Kellner et al., 2019; Khan et al., 2019; English et al., 2019; Sullivan et al., 2019; Luan et al., 2021; Wang et al., 2020; Zhou et al., 2020). While RPA/RAA is quicker and gives the advantage of working in the range of physiological temperature, it is more sensitive to contaminants and off-target nucleic acid contamination (Lillis et al., 2016; van Dongen et al., 2020; Aman et al., 2020). Loop-mediated isothermal amplification (LAMP) works at a range of 59–65 °C and is based on the mechanism of auto-cycling strand displacement DNA synthesis carried out by *Bst* DNA Polymerase. In contrast to PCR & RAA, LAMP uses two pairs of primers, one pair of inner and the other of outer primers, which are specially designed to carry out amplification through a cyclic reaction. The temperature-sensitive activity of Cas3, Cas9, Cas13, and Cas14 restrict their activity with RT-RAA, while on the other hand, LbCas12a, Aap-Cas12b, and Cas10-Csm complex have shown to have better thermo-tolerance and can be thus used with LAMP in single-pot assays (Broughton et al., 2020; Wang et al., 2021a; Li et al., 2019a; Joung et al., 2020; Santiago-Frangos et al., 2021). When RNA is used as starting material, there is an extra step to first convert it to cDNA through reverse transcription, which also can be mixed with PCR (RT-PCR) or isothermal amplification (RT-RPA/RT-RAA or RT-LAMP). A previous study showed that RPA can even be adapted with FnCas9-mediated nucleotide detection, eliminating the need for a PCR machine (Azhar et al., 2021).

Another isothermal amplification method that has been adapted for CRISPRDx is called Strand Displacement Amplification (SDA). This technique works at 60 °C and relies on a strand-displacing DNA polymerase, typically *Bst* DNA Polymerase, Large Fragment, or Klenow Fragment (3'-5' Exo), that initiates at nicks created by a strand-limited restriction endonuclease or nicking enzyme at a site contained in a primer. Unlike LAMP, it uses a single primer pair, and the nicking site is regenerated at each polymerase displacement step, resulting in exponential amplification (Zhou et al., 2018). Similarly, Nucleic Acid Sequence Based Amplification (NASBA) or Transcription Mediated Amplification (TMA) are isothermal amplification methods that require RNA as starting material. After an initial *in vitro* transcription step, an enzyme cocktail ensures RNA/DNA can be successfully amplified (Pardee et al., 2016).

Being a gRNA programmed RNA trans-cleavage effector, detection assays built around Cas13 protein require an extra step of converting DNA to RNA which is mostly done by T7 RNA polymerase (Gootenberg et al., 2017; Rauch et al., 2021). Since each of these nucleic acid amplification methods has its advantages and limitations, it becomes critically relevant to choose an amplification method that maximizes the sensitivity and specificity of the Cas effector. RPA/RAA is quicker, has

ease of primer design, and can be efficiently used as a single-pot. As a result, it has become the most practical choice for CRISPR-based diagnostic assays. However, the high sensitivity of RPA reaction components requires careful consideration of reaction conditions and handling to ensure non-specific amplification (Lillis et al., 2016). Unlike RPA, LAMP is more specific and shows flexibility to work with different readout methods and is also suitable for single-pot point-of-care diagnostics but at a non-physiological temperature range of 59–65 °C (van Dongen et al., 2020; Wang et al., 2015; Phillips et al., 2018). The choice of amplification methods covering traditional PCR or the isothermal alternatives is also linked with an individual Cas protein's activity at the temperature at which the amplification happens, especially for single-pot reactions. While isothermal alternatives like LAMP, NASBA, and SDA offer single-pot adaptability, not all the Cas proteins are tolerant to work in temperatures of 59–65 °C. Although other isothermal alternatives working near-physiological ranges like RPA/RAA have been adapted by several detection methods, the inconsistent outcomes can be of concern. The recent efforts towards direct sensitive detection without involving any pre-amplification of the nucleic acids can be really useful to avoid any bias introduced by or towards a particular amplification method. Some of such advancements to achieve direct detection or sensitive signal readouts are discussed in detail in the next section. Alternatively, isolation and characterization of thermotolerant Cas proteins or engineering Cas proteins for generating thermotolerant variants can expand the scope of such diagnostic methods beyond the ones currently used.

2.2. Different readouts used along with CRISPR-based diagnostics

Several diverse signal readouts such as fluorescence, colorimetric, lateral flow, microfluidics, electrochemical, and electronic have been employed for CRISPR-based nucleic acid detection assays. The most commonly used detection platform employs recording fluorescent intensities. Such an output is sensitive and ideal for Cas effectors that possess trans-cleavage activity. Here the reporters are usually fluorophore-quencher (FQ)-labeled single-stranded RNA/DNA or fluorophore-biotin (FB)-labeled single-stranded RNA/DNA. After the trans-cleavage of the non-targeted reporter probe, the readout is detected in terms of an increased fluorescence signal when the target is present (Chen et al., 2018; Bai et al., 2019; Xinjie Wang et al., 2020; Ding et al., 2020; Lucia et al., 2020; Broughton et al., 2020; Nguyen et al., 2021; Chen et al., 2018; Li et al., 2021; Wu et al., 2014; Wang et al., 2021a, 2021b; Liu et al., 2021; Teng et al., 2019; Li et al., 2019a; Guo et al., 2020; Joung et al., 2020; Harrington et al., 2018; Yoshimi et al., 2020; Gootenberg et al., 2017; Rauch et al., 2021; Zhang et al., 2020; Ackerman et al., 2020; Barnes et al., 2020; Hu et al., 2022; Fozouni et al., 2021; Gootenberg et al., 2018; Brogan et al., 2020). Although such detection methods are robust and convenient, the signals generated are relatively weak. Also, the availability of a fluorescence detector can be difficult in limited-resource settings, necessitating alternatives that are well-suited for point-of-care detection. Colorimetric assays on the other hand are more amenable to field settings. The majority of CRISPR-based assays are either coupled with biochemical readouts or use a UV/blue light source to detect fluorescence brightness produced during reporter trans-cleavage activity by Cas12, Cas13, and Cas14 CRISPR systems, Table 1. Additionally, it has also been observed that structured ssDNA reporters like hairpin-DNA and G-triplex DNA structures can perform better trans-cleavage, and thus increase the sensitivity of the reaction (Zhang et al., 2020; Li et al., 2021; Ke et al., 2022).

Another alternative point-of-care option is the use of lateral flow assays. LFAs typically have a visual readout, which can be obtained through the use of AuNPs (gold nanoparticles) on a paper-strip immunoassay that can capture FAM-labeled RNP (ribonucleoprotein)-bound or cleaved biotinylated substrate molecules as a distinct test line of the paper strip (Gootenberg et al., 2017; Xusheng Wang et al., 2020; Azhar et al., 2021; Bai et al., 2019; Lucia et al., 2020; Broughton et al., 2020;

Table 1

Summarizes major CRISPR-based nucleic acid detection assays developed to date. All characteristics are mentioned as claimed by the original publications, such as information about amplification method and signal readout used; Assays reported for single-variant or single-nucleotide detection are mentioned with Y, yes, and N, no; if not. *SpCas9* = *Streptococcus pyogenes* Cas9; *dSpCas9* = dead (catalytically inactive) *S. pyogenes* Cas9; *dFnCas9* = dead (catalytically inactive) *Francisella novicida* Cas9; *LbCas12a* = *Lachnospiraceae bacterium* Cas12a; *AaCas12b* = *Alicyclobacillus acidiphilus* Cas12b; *LwCas13a* = *Leptotrichia wadai* Cas13a; *LbCas13a* = *Leptotrichia buccalis* Cas13a; *LsCas13a* = *Leptotrichia shahii* Cas13a; *PspCas13b* = *Prevotella* sp. *P5-125* Cas13b; *PsmCas13b* = *Prevotella* sp. *MA2016* Cas13b; *TtCsm* = *Thermus thermophilus* Csm; NASBA, Nucleic Acid Sequences Based Amplification; RT-PCR, reverse-transcription polymerase chain reaction; RT-RAA, reverse-transcription recombinase aided amplification; RT-RPA, reverse-transcription recombinase polymerase amplification; RT-LAMP, reverse-transcription loop-mediated isothermal amplification; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; LFA, lateral flow assay.

Type	Cas effector	Assay Name	Pre-amplification Method	Signal readout	SNV/SNP detection	Reference
DNA targeting Cas systems	Type II <i>SpCas9</i> <i>dSpCas9</i> <i>dFnCas9</i> <i>LbCas12a</i>	NASBACC	NASBA	dsDNA cleavage/Toehold switch sensors	Y	Pardee et al. (2016)
		CAS- EXPAR	DNA pol. mediated primer extension	dsDNA cleavage/Real-time fluorescence	Y	Huang et al. (2018)
		CARP	Reverse-PCR	dsDNA cleavage/Real-time PCR	N	Zhang et al. (2020)
		CRISDA	SDA	dsDNA nickase/Fluorescence (Cy5)	Y	Zhou et al. (2018)
		CRISPR-Chip	Pre-amplification free	dsDNA binding/Graphene-based field-effect transistor (gFET)	N	Hajian et al. (2019)
		dSpCas9/SpCas9	SNP-Chip	dsDNA binding/Graphene-based field-effect transistor (gFET)	Y	Balderton et al. (2021)
		CADD	Pre-amplification free	dsDNA binding/Fluorescent hybridization chain reaction (HCR)	N	Xu et al. (2020)
		CASLFA	PCR or RPA	dsDNA binding/Lateral flow assay (Paper-strip)	N	Xusheng Wang et al. (2020)
		FELUDA	RT-PCR or RT- RPA	dsDNA binding/Fluorescence/ Lateral flow assay	Y	Azhar et al. (2021)
		HOLES	RPA	ssDNA trans-cleavage/ Fluorescence	N	Chen et al. (2018)
Type V	<i>LbCas12a</i> <i>opvCRISPR</i>	PCR or other isothermal amplification methods	RAA	ssDNA trans-cleavage/Fluorescence (HEX)	Y	Li et al. (2018)
		E-CRISPR	RAA	ssDNA tran-cleavage/ electrochemical biosensor	N	Dai et al. (2019)
		CORDS	RAA	ssDNA trans-cleavage/Lateral flow assay	N	(Bai et al., 2019)
		CRISPR/Cas12a-NER	RT-RAA	ssDNA trans-cleavage/ Fluorescence	N	Xinjie Wang et al. (2020)
		SCAN	Pre-amplification free	ssDNA trans-cleavage/Solid-state nanopore sensors	N	Nouri et al. (2020).
		AIOD-CRISPR	RPA	ssDNA trans-cleavage/ Fluorescence	N	Ding et al. (2020)
		–	RT-RPA	ssDNA trans-cleavage/ Fluorescence/LFA	N	(Lucia et al., 2020)
		SARS-CoV-2- DETECTR	Isothermal amplification	ssDNA trans-cleavage/ Fluorescence/LFA	N	Broughton et al. (2020)
		opvCRISPR	RT-LAMP	ssDNA trans-cleavage/ Fluorescence	N	Wang et al. (2021b)
		ENHANCE	RT-LAMP	ssDNA trans-cleavage/ Fluorescence/LFA	N	Nguyen et al. (2021)
AaCas12b	– <i>Cdetection</i> <i>HOLMESv2</i>	–	Pre-amplification free	hpDNA trans-cleavage/ Electrochemical reporters	N	Zhang et al. (2020)
		CASMEAN	RAA	ssDNA trans-cleavage/ Fluorescence	N	Chen et al. (2018)
		Cas12aFDet	PCR or RAA	ssDNA trans-cleavage/ Fluorescence	N	Li et al. (2021)
		–	Isothermal amplification	ssDNA trans-cleavage/ Fluorescence	N	Wu et al. (2014)
		–	Rapid PCR amplification	ssDNA trans-cleavage/ Fluorescence	N	Wang et al., 2021a
		–	PCR-amplified	ssDNA G-triplex structures trans-cleavage/Fluorescence/FLA	N	Li et al. (2021)
		–	RPA	ssDNA trans-cleavage/ Fluorescence	N	Liu et al. (2021)
		SERS Biosensor	LAMP	ssDNA trans-cleavage/surface-enhanced Raman scattering (SERS)	N	Pan et al. (2022)
		CLE-CRISPR	RPA	ssDNA trans-cleavage/ hybridization chain reaction (HCR) Chemiluminescence	N	Ke et al. (2022)
		Cas-PfLAMP	LAMP	ssDNA trans-cleavage/LFA	N	Zhu et al. (2022)
AaCas12b	– <i>Cdetection</i> <i>HOLMESv2</i>	–	Pre-amplification free	ssDNA trans-cleavage/dis-aggregation of gold nanoparticles/ color change	N	Ma et al. (2022)
		RT-RAA		ssDNA trans-cleavage/ Fluorescence	Y	Teng et al. (2019)
		RT-PCR or RT-LAMP		ssDNA trans-cleavage/ Fluorescence	N	Li et al. (2019a)

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Table 1 (continued)

Type	Cas effector	Assay Name	Pre-amplification Method	Signal readout	SNV/SNP detection	Reference
RNA targeting Cas systems	Cas14a	CASdetec	RT-RAA	ssDNA trans-cleavage/ Fluorescence	N	Guo et al. (2020)
		STOPCovid.v1	RT-LAMP	ssDNA trans-cleavage/ Fluorescence	N	Joung et al. (2020)
		STOPCovid.v2	RT-LAMP	ssDNA trans-cleavage/ Fluorescence	N	Joung et al. (2020)
	Type I	Cas14a	Cas14-DETECTR	RT-PCR or RT-RPA followed by T7 transcription	Y	Harrington et al. (2018)
		Cas3-Cascade	CONAN	Isothermal amplification methods	N	Shi et al. (2021)
	Type VI	LwCas13a	SHERLOCK	RT-PCR or RT-RPA followed by T7 transcription	Y	Gootenberg et al. (2017)
	LbCas13a	CREST	RT-PCR or RT-RPA/T7 transcription	RNA trans-cleavage/Fluorescence/ LFA	N	Rauch et al. (2021)
		SHERLOCK-COVID	RT-RPA followed by T7 transcription	RNA trans-cleavage/LFA	N	Zhang et al. (2020)
		CARMEN-Cas13	RT-RPA followed by T7 transcription	RNA trans-cleavage/Fluorescence	N	Ackerman et al. (2020)
		–	RT-PCR or RT-RPA followed by T7 transcription	RNA trans-cleavage/LFA	N	(Barnes et al., 2020)
		–	Pre-amplification free	RNA trans-cleavage/ Electrochemical microfluidic biosensor	N	Bruch et al. (2021)
Type V + Type VI	LbCas13a	ECS-CRISPR	RPA	RNA trans-cleavage/Fluorescence	N	Hu et al. (2022)
		–	Pre-amplification free	RNA trans-cleavage/Fluorescence	N	Fozouni et al. (2021)
		–	Pre-amplification free	RNA trans-cleavage/Droplet microfluidics	N	Tian et al. (2020)
	LbCas13a + TtCsm6	–	RT-RPA followed by T7 transcription	RNA trans-cleavage/catalytic hairpin DNA circuit (CHDC)	N	Sheng et al. (2021)
		FIND-IT	Pre-amplification free	RNA trans-cleavage in tandem/ chemically stabilized activator	N	Liu et al. (2021)
	LbCas13a + Cas14a	casCRISPR	Pre-amplification free	Cas13a trans-cleaved RNA and ST-HP activated trans-cleavage activity of Cas14a	N	Sha et al. (2021).
	LsCas13a	–	Pre-amplification free	Fluorescence/Light-up RNA aptamer	N	Zhang et al. (2020)
	LwCas13a + PspCas13b	CARVER	In vivo	–	N	(Freije et al., 2019)
	LbCas12a + LwCas13a + PsmCas13b + EiCsm6	SHERLOCKv2	RT-RPA followed by T7 transcription	RNA trans-cleavage/Fluorescence/ LFA	Y	Gootenberg et al. (2018)
Type VI	Cas13d (CasRx)	PAC-MAC SENS'R	In vivo RT-PCR or RT-RPA followed by T7 transcription	– RNA trans-cleavage/Fluorescence/ LFA	N N	(Abbott et al., 2020) (Brogan et al., 2020)
Type III	Cas10-TtCsm complex	–	RT-LAMP followed by T7-transcription	ssDNA/RNA Type III based colorimetric or fluorescence readouts	N	Santiago-Frangos et al. (2021)

Nguyen et al., 2021; Li et al., 2021; Yoshimi et al., 2020; Rauch et al., 2021; Barnes et al., 2020; Hu et al., 2022; Fozouni et al., 2021; Gootenberg et al., 2018; Brogan et al., 2020). While fluorescence detection using plate readers can adapt to increased throughput, paper-strip-based detection can be laborious when handling a large number of samples (Ackerman et al., 2020; Wang et al., 2020). For high throughput adaptations, electronic or electrochemical biosensors are better alternatives for signal readout. Indeed, some studies have reported assays that couple CRISPR nucleic acid detection with Graphene-based field-effect transistor (gFET) (Hajian et al., 2019; Balderston et al., 2021), toehold switch sensors (Pardee et al., 2016), hpDNA electrochemical reporters (Zhang et al., 2020; Sheng et al., 2021), and electrochemical microfluidic biosensor (Bruch et al., 2021). The use of electrochemical biosensors not only helps in increasing the throughput but because of their high sensitivity can detect substrates in the range of fM to aM (Zhang et al., 2020; Sheng et al., 2021). As shown in a few studies, the reusability or regeneration of electrochemical biosensor platforms can also help in reducing the waste generated through these detection assays

(Sheng et al., 2021).

Detection of more than one target in a single reaction or multiplexing samples gives the advantage of scale-up and low turn-around times for detection (Dincer et al., 2017; Wang et al., 2020). However, inherent noise between recognition molecules or various analytes coupled with possible cross-reactions can make it challenging to develop robust multiplex detection methodologies (Li et al., 2019b). SHERLOCK V2.0 (Gootenberg et al., 2018) was capable of multiplexing nucleotide detection by combining four different Cas effectors (PsmCas13b, LwCas13a, CcaCas13b, and AsCas12a) to detect four different targets in a single reaction. Here caution has to be taken for detecting distinct readouts from each Cas effector independently without affecting others. Multiplexing to quite an extent can also be done by tagging the type of Cas/sgRNA effector complexes with distinct fluorophores, which can allow simultaneous detection of targets and will be specific to fluorescent signaling only for matched sgRNAs (Osborn et al., 2021). Multiplexing through electrochemical biosensors or microfluidics gives numerous advantages but also requires a careful assay design, which can

be improved in the future with the discovery of more and more well-suited Cas effectors.

Even though significant advancements are made toward single-pot reactions, the nucleic acid amplification steps used for enhancing limited detection are associated with drawbacks such as increased assay time and the risk of cross or aerosol contamination. Addressing these, efforts are also being made to develop amplification-free CRISPR-based diagnostic assays either by opting for readouts that can amplify signal after Cas/crRNA effector activity or by combining multiple Cas effectors targeting several sites in a target sequence (Gootenberg et al., 2018; Freije et al., 2019; Ackerman et al., 2020; Liu et al., 2021). The increased signal sensitivity without needing an amplification step can be achieved by either of these means, by increasing the target concentration by reducing the reaction volume, using more sensitive electronic/electrochemical biosensors, or output signal magnification through a cascade or circuitry steps. This increase in local target concentration could be achieved by adapting to droplet microfluidics, wherein by the use of picoliter-sized systems or microchamber-array technologies, the detection sensitivity can be enhanced more than a thousand times achieving detection even to a single molecule. Along with being highly sensitive, such systems enable absolute target nucleic acid quantification (Tian et al., 2020; Bruch et al., 2021; Yue et al., 2021; Shinoda et al., 2021).

Detection through electrochemical sensors, which are compact and highly sensitive, can be another means of enhancement of sensitivity. For example, E-CRISPR (CRISPR Cas12a-based electrochemical biosensor) uses a three-electrode sensor having an ssDNA reporter linked with methylene blue (MB) for detecting Cas12a trans-cleavage activity (Dai et al., 2019; Li et al., 2021). As mentioned before certain DNA morphological structures can be efficiently trans-cleaved with Cas12a and when combined with electrochemical detection strategies, can be highly sensitive (Zhang et al., 2020; Lee et al., 2021). Direct detection using a glass nanopore sensor has also shown promising signal magnification, detecting single molecules without involving any nucleic acid amplification (Nouri et al., 2020). For detecting multiplexed targets, the integration of an electrochemical biosensor with microfluidics can allow amplification-free quantification of multiple target RNAs simultaneously (Bruch et al., 2021). The E-DNA or CRISPR-based enhanced electrochemical DNA system uses target DNA activator molecules annealed with an ssDNA signaling probe, as a double-stranded DNA reporter for Cas9-or Cas12a-mediated cis-cleavage giving out a signal through separation of electrochemical tag from the probe (Xu et al., 2020). On the other hand, a combination of catalytically-inactive, dead Cas9 (dCas9) with the graphene-based field-effect transistor, namely CRISPRChip is shown with a 15-min rapid and sensitive detection of unamplified genomic DNA (Hajian et al., 2019).

To increase the signal strength, early attempts were made by combining different Cas effector activities to generate Cas-based cascade signal amplification. For instance, the combination of Csm6 RNA endonucleases complex with Cas13a in the improved SHERLOCKv2 showed better CRISPR detection sensitivity with Cas13a alone (Gootenberg et al., 2018). This was recently improved as FIND-IT, utilizing chemically stabilized activators to be targeted directly by Cas13a followed by the action of activated Csm6 RNA endonucleases onto RNA reporters to produce a fluorescent signal (Liu et al., 2021). Similarly, with the help of a specially designed ST-HP (locked-trigger for Cas14a/sgRNA with two uracil ribonucleotides in the loop) activator, the casCRISPR (cascade CRISPR/cas) system works in combination by utilizing the trans-cleavage products obtained after Cas13a activity for Cas14a activation and triggers trans-cleavage of reporters for the signal magnification. This combination of the casCRISPR system can improve the detection limit by 1000 times (1.33 fM) compared to when Cas13a is used alone (Sha et al., 2021). The use of a Cas effector to create a positive feedback circuit is another way of trans-cleavage signal amplification. In the method named CONAN, two crRNAs get simultaneously paired to the target nucleic and an assistant probe. The assistant crRNA paired

with ssDNA FQ-reporter can be only released after the trans-cleavage of ssDNA blocker on finding the target nucleic acid sequences. This results in one-step signal amplification through Cas12a-mediated trans-cleavage of more FQ-reporters and real-time attomolar level detection in a probe-guided positive feedback manner (Shi et al., 2021). Simultaneous cleavage at multiple sites within the target nucleic acid sequences can also be a way to achieve signal amplification: a combination of three Cas13a/crRNA complexes targeting the SARS-CoV-2 genome was recently reported for direct and amplification-free detection of SARS-CoV-2 with ~100 copies/µL sensitivity (Fozouni et al., 2021). Much recently amplification-free Cas12a nucleic acid assays were developed by coupling ssDNA collateral cleavage with more sensitive readouts such as surface-enhanced Raman scattering (SERS) and dis-aggregation of gold nanoparticles to achieve a single molecule direct DNA detection (Pan et al., 2022; Ma et al., 2022).

Together with qualities like rapid, single-pot reaction, and increased throughput, CRISPDx assays have recently become amenable to automation (Kellner et al., 2019; Ackerman et al., 2020; Ramachandran et al., 2020; Palaz et al., 2021). During the last 5–6 years, CRISPR-based nucleic acid detection assays have been modified, improvised, and tailored for diverse applications such as pathogenic or infection diagnostics, agriculture, and food safety, genotyping of viral and bacterial strains and industrial biotechnology, etc (Zuo et al., 2017; Chiu, 2018; Chertow, 2018; Li et al., 2019b; Abudayyeh et al., 2019; Abudayyeh and Gootenberg, 2021; Zhu et al., 2022; Mu et al., 2022; Mao et al., 2022; Qian et al., 2022; Hu et al., 2022).

3. Cas systems with nucleobase discrimination and possible application

The discovery that various Cas systems can discriminate between sequences having a single base change has expanded the toolkit for SNV/SNP detection and subsequent interrogation of such genetic variation. Rapid and early detection of genomic alterations is critical in clinical diagnosis. Genomic alterations are mostly confounded with SNVs, which are either directly associated with disease phenotypes or have causal roles in disease progression. Researchers are now trying to harness the single base specificity of several CRISPR systems to develop POC diagnostics that can easily discriminate SNV across DNA/RNA substrates. Initial work on SNV detection via the Cas system relied on the presence of mutated/mismatched nucleotides at the cleavage site or within the PAM sequence (Pardee et al., 2016; Zhou et al., 2018; Hajian et al., 2019). The mutation in the PAM sequence leads to disruption of the initial PAM-dependent binding of Cas effector and can thus help differentiate between wild-type and mutated sequences but as PAM is not always present at the target DNA/RNA sequences, their applicability for diagnostics assays is limited. The first-ever SNV detection, through target sequence mismatch sensitivity, was reported using Cas13a (Myhrvold et al., 2018). Cas13a was used along with a crRNA designed in such a way that the single-nucleotide polymorphism (SNP) site was at the third position of the spacer sequence and the synthetic mismatch was placed at the fourth or fifth position of the spacer sequence. This technique could successfully detect a single-nucleotide mismatch between African and American strains of the Zika virus, Fig. 3. Since then, various CRISPR-based SNV detection platforms have been developed using different Cas effectors, and multiple detection readouts. These have been summarized in Table 2 and explained briefly in Fig. 3. An important consideration to make in every SNV detection method is that the gRNAs generated with mismatched nucleotides can potentially develop a fresh set of potential off-targets depending on the base-pairing off-target sequences and altered gRNA. Thus, one should be cautious to check for off-targets when using altered gRNA for *in vitro* as well as *in vivo* SNP detection.

The CRISPR-based SNV detection platforms have improved in terms of sensitivity to mismatches in the seed sequence. For example, Cas14a can discriminate between human *HERC2* gene DNA substrates having an

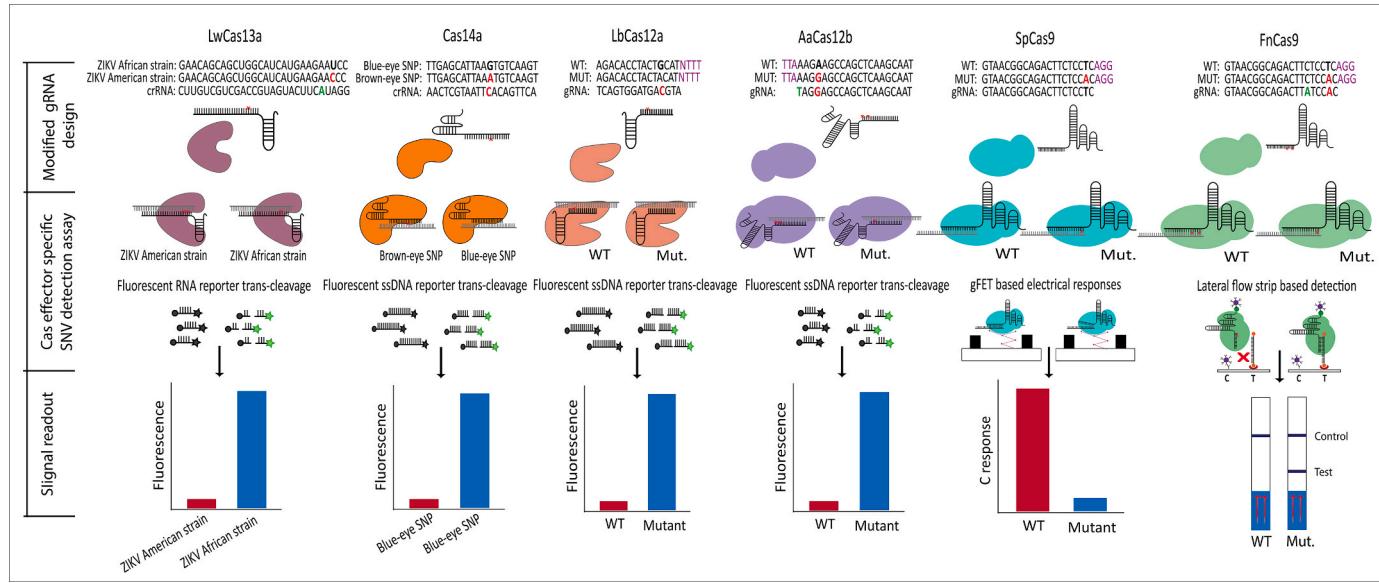


Fig. 3. Different CRISPR-based SNV detection methods shown with examples of gRNA designs and typical signal readouts previously reported. The Cas effectors such as LwCas13a, Cas14a, LbCas12a, and AaCas12b which show differences in their trans-cleavage activities upon interacting with an SNV provide ease to be combined with fluorescence reporter cleavage readouts but the interference produced in the target binding of SpCas9 or FnCas9 due to presence of an SNV make them more suitable for binding based readouts like LFA or gFET, etc. The signal differences between WT and mutant are purely qualitative based on the previously published reports. WT nucleotide is shown in black bold while mutant nucleotide is in red. Violet nucleotides represent PAM and green corresponds to synthetic mismatches in the gRNA used for detection.

Table 2

Different Cas systems reported for SNV/ SNP detection through target mismatch sensitivity. All information including mismatched sensitive positions is obtained from the original publications. *The requirement of PFS for LwCas13a is not mandatory but can improve cleavage efficiency; *Cas14a requires T-rich TTTA PAM sequences only when cleaving dsDNA but no PAM is required for ssDNA cleavage; *Mentioned AaCas12b crRNA mismatched nucleotide positions in the table are shown with maximum sensitivity, other crRNA positions with moderate or low sensitivity are also mentioned in the original publication. LbCas12a, Cas14a, and SpCas9 are shown to discriminate single-nucleotide changes without any synthetic mismatch nucleotide while LwCas13a, AaCas12b, and FnCas9, require an additional synthetic mismatch nucleotide working in combination for the SNV/SNP detection.

Cas effector	PAM/ PFS	Spacer RNA	Cleavage target	Mismatch-sensitive positions (From PAM)	Reference
LwCas13a	NA*	22–28	ssRNA	3&4, 3&5	Myhrvold et al. (2018)
LbCas12a	TTTN	17–18	ssDNA/ dsDNA	1st -7th	Li et al. (2018)
Cas14a	NA	20	ssDNA/ dsDNA*	11&12, 12	Harrington et al. (2018)
AaCas12b*	TTN	20	ssDNA/ dsDNA	1&4, 1&5, 4&11, 4&16, 5&8, 5&11, 16&19*	Teng et al. (2019)
SpCas9	NGG	20	dsDNA	2, 3	Balderston et al. (2021)
FnCas9	NGG	20	dsDNA	2&6, 16&19	Azhar et al. (2021); Kumar et al. (2021)

SNP responsible for eye color ([Harrington et al., 2018](#)). In this assay which got referred to as DNA endonuclease-targeted CRISPR trans reporter or DETECTR assay, HERC2 DNA substrate from human saliva of blue-eyed and brown-eyed individuals was amplified using primers containing phosphorothioate (PT) such that one strand remains protected from degradation by exonucleases. Hence, upon the addition of

T7 exonuclease, the unmodified strand is degraded, leaving ssDNA substrates that can be analyzed for the presence of different SNPs by Cas14a, [Fig. 3](#). Moreover, Cas14a is known for its PAM independence activity and can therefore have an exhaustive list of targets. Both LwCas13a and Cas14a have the trans-cleavage ability *in vitro* i.e., after binding to a specific target, these get activated to cleave DNA/RNA fragments nonspecifically. This characteristic feature has been exploited to develop detection readouts such as fluorescently labeled reporters that also help in the amplification of the signal. While LwCas13a has been reported to show mismatch sensitivity at PFS proximal 3rd and 4th or 3rd and 5th positions, a thorough study to validate all the possible combinatorial positions which can be exploited for SNV detection is required. The RNA-targeting LwCas13a still requires an additional step of DNA to RNA conversion. On the other hand, Cas14 which works directly on ssDNA/dsDNA requires a TTTA PAM for the dsDNA cleavage but shows no PAM constraint when used against ssDNA as targets. Because of this PAM flexibility even with a limited number of mismatch-sensitive positions, Cas14a can target SNVs to a greater extent when compared to other Cas effectors ([Harrington et al., 2018; Ansari et al., 2022](#)).

To date, several subtypes of Cas12 proteins have been discovered ([Yan et al., 2019; Makarova et al., 2020](#)). Among these, LbCas12a, requiring T-rich PAM sequence (TTTN), has been shown to have mismatch sensitivity towards single-nucleotide mismatches present within the PAM or seed region (1st-7th base, PAM proximal) of the spacer sequence. In addition, with the use of shorter gRNAs (17-18 nt), higher cleavage specificity was observed that could enhance the detection of mismatches. Thus, this Cas12a-based assay, commonly known as HOLMES can work with a broader window of mismatch sensitivity. When tested against human saliva samples, HOLMES was successful in detecting the presence of gout disease-related SNP rs1014290 (G > A), [Fig. 3](#), and could even distinguish between homozygous and heterozygous alleles ([Li et al., 2018](#)). Similarly, Cas12b, when used with a tuned guide RNA (tgRNA) that contained a single-nucleotide mismatch in the spacer sequence could efficiently distinguish between two target sequences varying by a single base ([Teng et al., 2019](#)). It has been used to determine blood types with high accuracy. Moreover, the Cas12b-based

detection platform can retain its mismatch sensitivity with several combinatorial positions in the spacer sequence, thus opening up a large window for detecting single-base mutations in the human genome. As a proof of concept, it could distinguish mutations such as cancer-related TP53 (856G > A) and breast cancer-related BRCA1 gene (3232A > G and 353 7A > G), Fig. 3. Again, this platform works based on increased fluorescence in the case of SNP as compared to a near-background signal for the wild-type alleles. Thus, it can rapidly detect DNA at the single-base resolution for clinical diagnostics. However, because of its inability for the trans-cleavage activity that is owed to its tight binding with the cleaved DNA targets, a pre-amplification step for detectable signals is necessary (Teng et al., 2019).

Although both *LbCas12a* and *AaCas12b* have been reported with several crRNA mismatch sensitive positions for SNV detection, due to differences in the PAM dependability they both can show differences in the number of SNVs that can be targeted. This corroborates that a Cas effector with relaxed PAM shows better targeting options. Even though Cas12b can target SNVs, the increased sensitivity towards ssDNA trans-cleavage by Cas12a has been opted for by numerous diagnostics assays. To reduce the PAM dependability of *LbCas12a*, a PAM sequence ("TTTN") could be introduced within the primers used for PCR amplification, allowing it to detect SNVs in a PAM sequence-independent manner (Li et al., 2018).

Just like other Cas systems even the canonical Cas9 possess sensitivity to mismatches within the PAM proximal crRNA positions. But because of its strong binding towards the target or off-target dsDNA sequences, the reliable differences for SNV detection can only be achieved through adapting to many sensitive readouts. This is what has been shown with the use of a highly sensitive Graphene-based field-effect transistor (gFET) for CRISPR-based single nucleobase discrimination (Balderston et al., 2021). Due to the use of gFET-based readouts, even the slightest difference in Cas9 binding towards wild-type and mutant sequences can be enhanced and detected as multiple types of electrical responses. This detection platform, which is named the SNP-Chip, works by anchoring catalytically inactive Cas9 protein to graphene monolayer and is subjected to unamplified dsDNA molecules to record the differences in C-response in real-time, Fig. 3. Other than having a sensitivity of 6.3 fM, this method of SNP detection is quite rapid, cheap, and compact, which can be advantageous over others. However, it requires dedicated instrumentation that can limit its usage in limited-resource settings. Similar to SNP-Chip, recently reported CARVE-seq also utilizes spCas9's ability to single-nucleotide discrimination to accurately detect low-frequency SNVs by combining spCas9 cleavage with a label-based molecular barcodes strategy before the library preparation for sequencing (Lee et al., 2021). The CARVE-seq again highlights the importance of PAM integral and proximal positions for spCas9 cleavage in SNV detection.

Recently, another orthologous CRISPR/Cas9 system from the bacteria *Francisella novicida* (FnCas9), has been reported to have single-nucleotide mismatch sensitivity (Azhar et al., 2021). It was observed that sgRNAs having mismatches at defined positions mismatched sgRNAs obligated FnCas9 association to the target sequences and thus compromised the binding of FnCas9 with the target. FnCas9 could not bind or cleave targets having two mismatches at the 2nd and 6th position (PAM proximal) of the spacer. Thus, SNP at either of these positions could be detected through an additional synthetic mutation in the sgRNA at the other position. The ability to discriminate SNPs due to the varying binding affinity of FnCas9 was utilized to develop a non-cleavage, affinity-based assay called FELUDA (FnCas9 Editor Linked Uniform Detection Assay), Fig. 3. With a strategic sgRNA designing approach, FELUDA was deployed for the detection of causal SNV (GAG > GTG) for sickle cell anemia (SCA), wherein it could efficiently distinguish between the samples consisting of homozygous (SCA) and heterozygous alleles (SCT). FELUDA with the use of a catalytically dead FnCas9 (dFnCas9) can also be combined with multiple readouts like fluorescence-based detection, lateral flow assay (LFA), etc. (Azhar

et al., 2021).

To increase the possibilities for rapid diagnostics during the current COVID-19 pandemic, many previously reported CRISPR/Cas-based nucleic acid detection assays were either reprogrammed or adapted to enable point-of-care detection for SARS-CoV-2 infections. Just like SHERLOCK, DETECTR, STOPCovid, HOLMESv2, etc. (Broughton et al., 2020; Joung et al., 2020; Guo et al., 2020; Ding et al., 2020; Wang et al., 2021a; Fozouni et al., 2021), FELUDA was also repurposed as a CRISPR-based lateral flow assay (LFA), which allowed for an accurate, low-cost SARS-CoV-2 detection assay, free of complex instruments (Azhar et al., 2021). Because of their quick deliverables in health emergencies, many of these CoV-2 detection assays were even given Emergency Usage Authorization (EUA) and commercialized rapidly for public use. The ongoing COVID-19 pandemic has worsened owing to rapid mutations within the SARS-CoV-2 genome. Several of these mutations have also been shown to be associated with higher transmission, immune escape, and disease severity. Tracking these variants has also become significant in understanding how the virus propagates and responds to vaccines. Currently, high throughput sequencing of complete viral genomes is the best available method to gain insight into the evolution of viral mutations and their subsequent risk stratification. However, the use of sequencing techniques as a generalized diagnostic method to detect various SARS-CoV-2 variants is expensive, requires specialized infrastructure and trained personnel, and has a long turn-around time (Vandenberg et al., 2021; Jayamohan et al., 2021).

To tackle this many groups tried to develop or repurpose different CRISPR-based SNV/SNP detection platforms for the rapid detection of various SARS-CoV-2 variants in a cost-effective manner, summarized in Table 3. One such recently developed paper-strip-based SNV/SNP detection platform called Rapid variant AssaY (RAY), is an adaptation of the previously reported FELUDA method. Using a larger window for mismatch discrimination RAY can provide visual readouts that show the presence of a variant signature within a CoV-2 sample within an hour. RAY thus not only successfully detects patients positive for SARS-CoV-2 infection, but also identifies an evolved and highly transmitted N501Y variant of CoV-2 lineage. Using this simple strategy of paper-based CRISPR diagnostics several CDC-listed major VOCs/VOIs that lie within the window of FnCas9 sensitivity could be detected, making RAY a valuable asset which can be appointed as a primary surveillance method for isolating cases infected with evolved lineages (Kumar et al., 2021).

Similarly, the previously reported DETECTR assay was recently repurposed around a newly identified and more specific CRISPR-Cas12 enzyme called CasDx1, to CRISPR-based COVID-19 variant DETECTR assay. This utilizes fluorescent detection using a CRISPR-Cas12 enzyme after an RT-LAMP pre-amplification. In combination, they could show SNP discrimination for spike protein amino acid-related mutations at positions 452, 484, and 501 (Fasching et al., 2021). Another CRISPR-Cas13 transcription amplification method, relying on Cas13a-based sequence specificity and Light-up RNA aptamer for signal amplification could perform nucleobase discrimination for SARS-CoV-2 D614G variants and can be designed to detect other respiratory viruses (Wang et al., 2021a,b). The single-base sensitivity of Cas12a in combination with a reverse transcription-PCR (RT-PCR) with one of the amplification primers introducing PAM near any target SNV could be a universal SNV targeting adaption. Utilizing an assay reported by Liang et al. (2021) could readily detect several spike protein mutations such as K417N/T, L452R/Q, T478K, E484K/Q, N501Y, and D614G, and could discriminate between several major SARS-CoV-2 variants (Liang et al., 2021). Recently repurposed *LbCas12a*-based miSHERLOCK combines optimized one-pot SHERLOCK reaction with an RNA capture through a PES membrane directly from a person's saliva. Once the RNA-containing PES membrane is plunged into a reaction tube underneath, an *in situ* nucleic acid amplification occurs together with Cas-based fluorescent output. This is quantifiable by a smartphone application. With the miSHERLOCK platform, RNA isolation and detection could be

Table 3

Different CRISPR-based nucleic acid detection assays readily repurposed during SARS-CoV-2 pandemic for SNV detection using Cas effector's ability to discriminate single-nucleotide changes.

Cas effector	Repurposed Assay Name	Pre-amplification Method	Signal readout	SARS-CoV-2 variants detected	Reference
dFnCas9	RAY	RT-PCR	dsDNA binding/Lateral flow assay	Multiple variants but individually	Kumar et al. (2021)
CasDx1	COVID-19 Variant DETECTR	RT-LAMP	ssDNA trans-cleavage/Fluorescence	L452R, E484K/Q/A, and N501Y	Fasching et al. (2021)
LwCas13a		Ligation-triggered transcription	RNA trans-cleavage/Light-up RNA aptamer	D614G	Wang et al. (2021b)
LbCas12a		RT-PCR	ssDNA trans-cleavage/Fluorescence	K417 N/T, L452R/Q, T478K, E484K/Q, N501Y, and D614G	Liang et al. (2021)
LbCas12a	miSHERLOCK	RT-RPA	ssDNA trans-cleavage/Fluorescence	N501Y, Y144del, and E484K	de Puig et al. (2021)
LbCas12a	RT-CORDS	RT-PCR	ssDNA trans-cleavage/Fluorescence	69/70 deletion, N501Y, and D614G	He et al. (2022)
LwCas13a & LbCas12a	mCARMEN	RT-PCR	RNA and ssDNA trans-cleavage/Fluorescence	Multiple viruses and variants simultaneously	Welch et al. (2022)

performed at a single spot, improving sensitivity and decreasing aerosol contamination. The modular miSHERLOCK can also be adapted to a multiplexed detection not just for SARS-CoV-2 and its variants but also for the other viruses and variants of concern (de Puig et al., 2021). Lately, another adaptation of Cas12a called Cas12a-based RT-PCR combined with CRISPR on-site rapid detection system (RT-CORDS) platform, could show detection of key mutations present in SARS-CoV-2 variants, such as 69/70 deletion, N501Y, and D614G. Relying on SNV-specific crRNAs with mismatches to differentiate between wild-type as well as mutant sequences, RT-CORDS have a detection limit of 6 copies/μL (He et al., 2022). Another recent adaptation was made to CARMEN by combining previous CRISPR-based diagnostics with microfluidics, such as microfluidic CARMEN (mCARMEN). This uses a combination of Cas13 and Cas12 with a microfluidics readout enabling quantitative measurement of viral copies along with high-throughput surveillance of multiple viruses and variants at once (Welch et al., 2022). Not just the detection or discrimination of SARS-CoV-2 and its variants but these CRISPR-based rapid SNV detection can be real assets for monitoring resistance towards infectious disease. For example, the SHERLOCK platform utilizing LwCas13a can perform species-specific detection by detecting the presence of A581G single nucleotide variant in dihydropteroate synthetase (dhps) associated with *P. falciparum* sulfadoxine resistance (Cunningham et al., 2021).

All together CRISPR-based SNV/SNP detection or discrimination has added a new dimension to CRISPRDX and can be utilized for a plethora of applications in various fields. For example, Cas12b-based rapid SNV/SNP detection assay reported for cancer-related *TP53* (856G > A) and breast-cancer-related *BRCA1* gene (3232A > G and 353 7A > G) can aid early screening for patients who might develop breast cancer later in their life (Teng et al., 2019). Likewise, multiplexed CRISPRDX platforms can detect several disease-related point mutations at once. A curated database of CRISPR-based SNP detection tools across different genomes can help us understand the targetable span for mismatch-sensitive Cas effectors reported till now (Teng et al., 2019). Among other applications, CRISPR-based nucleotide mismatch sensitivity has also been used to discriminate between methylated and unmethylated DNA sequences (Huang et al., 2018). After the bisulfite-treated conversion of cytosine residues to uracil in a target ssDNA sequence, the unaltered 5-methylcytosine recognized by gRNA gives cleavage outcomes. Consequently, CAS-EXPAR could show sensitivity toward a U-G mismatch indicating that the single cytosine methylation could be distinguished by CAS-EXPAR (Huang et al., 2018).

4. Concluding remarks and future perspectives

Usually different disease-associated point mutations are either detected through sequencing or nucleic acid probe-based hybridization.

However, sequencing is laborious, time-consuming, costly, and requires trained personnel, while probe-based assays show generally poor selectivity for SNV detection. Furthermore, the accuracy of SNV detection or variant calling by any sequencing platform is highly dependent on the depth and coverage of the reads to the reference genome. Due to the unavailability of simple, low-cost, rapid, and robust detection methods, people have relied on next-generation sequencing (NGS) for genotyping diseased mutations. However, the past few years have witnessed enormous growth in applications of the CRISPR/Cas systems and granted multiple Emergency Usage Authorizations (EUA), showcasing the power associated with the abilities of CRISPRDX. The field is currently not just limited to primarily gene editing but has expanded well into nucleic acid detection assays. The rapidness and ease with which the various CRISPR-based nucleic acid detection platforms were developed, or repurposed, was evident during the ongoing COVID-19 pandemic. High nucleobase specificities associated with these CRISPR/Cas systems have allowed us to utilize CRISPRDX for SNV detection (Kumar et al., 2021; de Puig et al., 2021; Wang et al., 2021b; Fasching et al., 2021; Liang et al., 2021; He et al., 2022; Welch et al., 2022). Moreover, due to the flexibility of multiplexing various Cas systems, one can design microchip panels by simultaneously placing multiple mutation-scanning sgRNAs for a rapid readout from patient samples, Fig. 4a (Ackerman et al., 2020). Additionally, by using four different fluorescently tagged Cas effectors with respective mismatched nucleotides gRNAs, nucleobases at a given position within an RNA/DNA sequence can also be determined. Once designed strategically, such assays can be beneficial for CRISPR-associated affinity-based target sequencing, Fig. 4b.

Even though CRISPR-based nucleobase detection/discrimination has opened up newer applications, it has certain shortcomings that need improvement. Currently, the prime disadvantage of this technique is the limited number of targetable mismatch positions/combinations that can be detected using the available Cas effectors. The added necessity of a PAM/PFS at a fixed locus from the position of the target SNV also limits the total number of targets that can be detected. A more systematic array of different combinations of sgRNA/spacer sequences that might be sensitive to SNV could be done for all the Cas effectors. Moreover, engineering PAM-flexible or PAM-independent Cas effectors, or searching for effectors sensitive to more permutations of mismatched positions are other crucial aspects that increase the targetable regions in the genome (Kleinsteiner et al., 2015; Chatterjee et al., 2020; Ansari et al., 2022; Zhu et al., 2022). Thus, Cas effectors with no or fewer PAM constraints can increase their SNV targeting abilities across different genomes. On the other hand, a stringent PAM can have the advantage of reduced off-target effects and could be a trade-off choice between the ability to target an SNV or off-targeting by a Cas effector.

Multiplexing CRISPR assays that can identify multiple targets in a

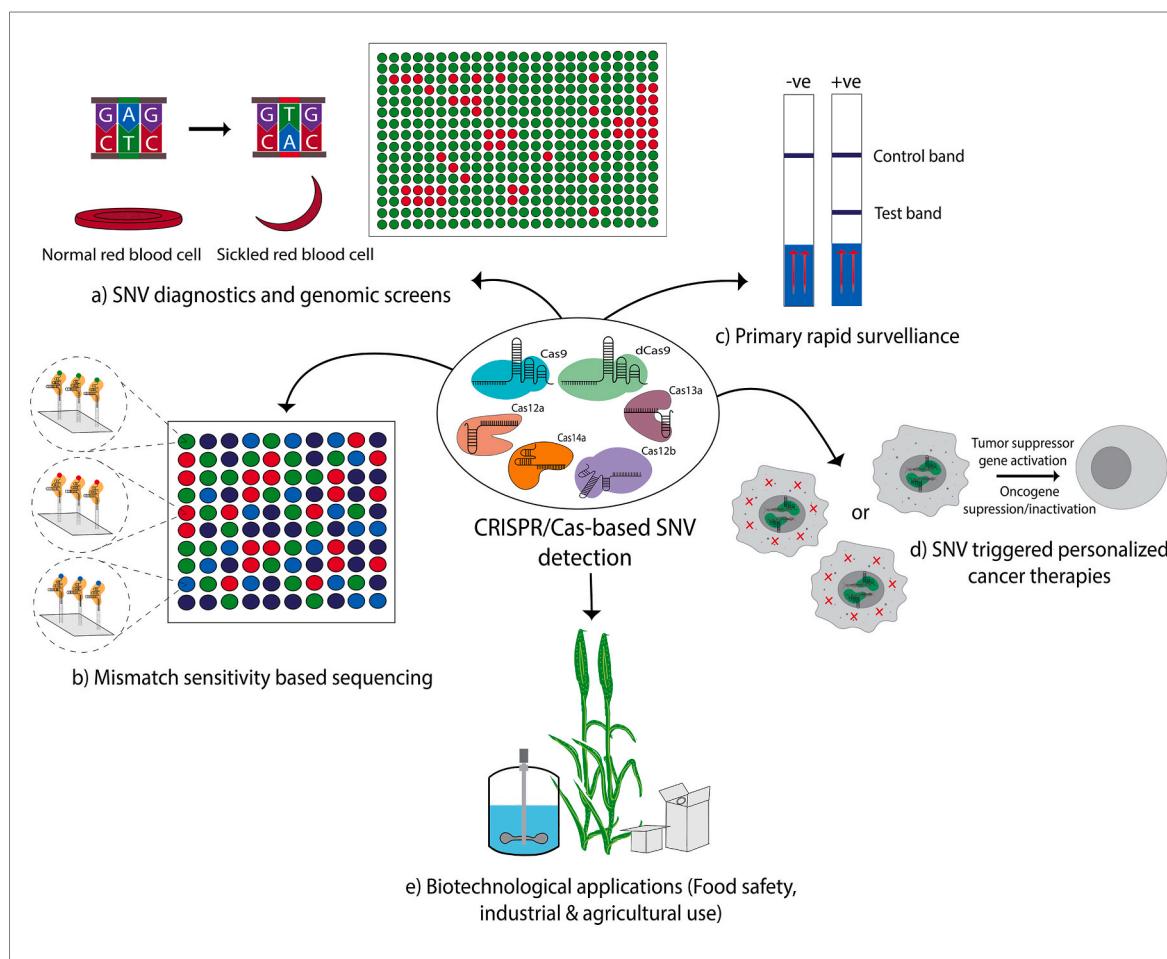


Fig. 4. Possible applications associated with rapid CRISPR/Cas-based SNV detection/discrimination assays **a)** SNV diagnostics and genomic screens can be used to screen pathogenic SNVs across a large number of patient samples. Also, to perform genome level screens to detect the presence of various SNVs which are to be assayed at once. **b)** Mismatch sensitivity-based sequencing through four different fluorescently tagged Cas effectors with respective mismatched nucleotides gRNAs, to identify nucleobases at a given position within RNA/DNA sequences. **c)** Primary rapid surveillance through CRISPRdx-based variants detecting visible readout assays. **d)** SNV-triggered personalized therapies, leading to targeted cell death via activation of suicidal genes or to limit cancer progression via tumor suppressor gene rescue or oncogenic gene repression. **e)** Biotechnological applications, rapid assays to detect mutant bacteria and their fitness, associated with industrial or agricultural importance.

single reaction provide advantages like rapid turnaround time and initial low sample requirement (Gootenberg et al., 2018; Ackerman et al., 2020; Welch et al., 2022; Li et al., 2022). However, inappropriate noise between recognition molecules or various analytes and possible cross-reactions can make it challenging (Wang et al., 2020). Therefore, careful reaction composition and design that is suitable for different Cas effectors has to be considered. The availability of different fluorophores to some extent can also help in expansion and performing multiplexed detection assays. Prior requirement of sequence information is another major constraint for CRISPR-based detection platforms as sgRNA designing requires target sequence information. Deep-sequencing on the other hand can provide a de novo sequence of novel organisms and thus remains valuable for discovering new mutations and identifying how variants evolve in humans or other organisms (Chiu and Miller, 2019). On the contrary, if the sequence information is already available, the CRISPR-based SNV detection platform can be more favorable as a quick diagnostic tool for the early detection of variants in a viral or bacterial infected sample (Wang et al., 2020; Chakraborty et al., 2021). As shown for Cas13a-based ZIKA viral strain identification and FnCas9-based rapid paper-strip diagnostic tool capturing major CoV2 VOC/VOIs can be useful in determining the source variant in the sample and which further can allow for opting for preventive measures to restrict the highly transmissible viral variants and can help in keeping a tight rein on

transmission-based evolution of virus during health emergencies, Fig. 4c (Pardee et al., 2016; Kumar et al., 2021; Dronina et al., 2022). Such rapid assays can also be important for low-cost and robust detection of resistance-inducing viral mutations, which can help in assessing the proper medications against infectious diseases (Cunningham et al., 2021).

Another limitation is the absence of a unified designing web tool that can help users with a basic understanding of CRISPR systems to quickly design and implement CRISPR-based single nucleotide detection assays for a target SNV. As listed previously in Table 2, different Cas effectors show mismatch tolerance at different spacer positions, which makes it even more laborious to assess all the possible combinations and possibilities for a particular. JATAYU (Junction for Analysis and Target Design for Your FELUDA assay), is a web-based tool that currently works with the idea of harnessing the single nucleotide mismatch tolerance ability of FnCas9 to develop large-scale screening assays. Here, by providing a sequence for any human SNV, the user can quickly design and implement a nucleic acid screening assay for that SNV across samples. JATAYU provides sequences for both the FnCas9 sgRNA and flanking amplification primers for the provided SNV (Azhar et al., 2021). Similarly, this database can be integrated with information about the mismatch tolerance ability of other Cas effectors. Such web-based databases can augment users in the rapid development of CRISPR-based

rapid SNV detection assays. A recent upgrade of JATAYU to another web server CriSNPr (CRISPR-based SNP recognition) eventually does that by integrating previously published information about multiple CRISPR-based SNV detection platforms and provides the user with modified gRNAs along with ready-to-use oligonucleotide sequences based on six CRISPRDx proteins of choice (Fn/enFnCas9, LwCas13a, LbCas12a, AaCas12b, and Cas14a) for SNV detection in relevant samples (Ansari et al., 2022). Even though tools like CriSNPr are vital in reducing efforts to design CRISPR-based SNV detection assays, a scoring algorithm-generated on previously published SNV-detecting gRNA designs can provide the user with a selectivity option to find the best gRNA designs. Unfortunately, to date, there is not enough experimentally validated data that can be used to build such a scoring algorithm, and till then the alternative would be to try different gRNA designs depending on the individual Cas effector's SNV targeting ability. Recent advancements are made through machine learning algorithms to predict Cas13a sgRNA efficiency both either by its ability to find a protospacer flanking site (PFS) and the mismatch-sensitive 'seed' region (Guo et al., 2021) or by predicting collateral activity through a high-throughput model to develop highly sensitive gRNA designs with better enzymatic activity (Metsky et al., 2022). Such algorithms have shown superior results to achieve highly sensitive gRNA designs but they are yet to be developed for gRNA design considering CRISPR-mediated SNV detection. Future development in algorithms predicting change in enzymatic activity from reported mismatch-sensitive gRNA designs can help in better design assessment and selection of best nucleotide positions to be used for consistent results.

The development of *in cellulo* CRISPR-based SNV detection through biochemical activities for signal readouts has not been fully explored yet. In this CRISPR-based, SNV detection that is linked to signal output through the activation of proximity ligation assays can be attempted. A simple example would be to visualize DNA/RNA using CRISPR probes inside cells with high resolution. These assays can be similarly performed in the pathology lab to microscopy studies and with automation can give rise to accurate multiplexable readouts. Importantly, these can be extended to tracing or studying pathogenic nucleic acids in sub-populations of cells in a live interface. Once a single system can be multiplexed with the detection of various SNVs through multiple Cas effectors such a detection method can further allow us to configure readout dynamics.

Cas12a-mediated DETECTR, FnCas9-mediated FELUDA/RAY and Cas13a-mediated SHERLOCK SNV detection platforms have been used for developing affordable, point-of-care diagnostics for the detection and monitoring of SNVs (Li et al., 2018; Azhar et al., 2021; Kumar et al., 2021; Kellner et al., 2019; de Puig et al., 2021). SNV-targeted CRISPR as a therapy can be an attractive possibility for the future, where the presence of a driver SNV would trigger a suicidal signal or cause a cell to kill itself through apoptosis upon CRISPR activation, Fig. 4d. Similarly, SNV in the gene can activate CRISPR-based changes at the target locus leading to the rescue of a tumor suppressor gene or repression of an activated oncogenic gene, Fig. 4d. Selective elimination of cells with mutations while leaving healthy cells unharmed is a personalized therapeutic strategy that might become feasible as CRISPR systems become more and more precise and their ability to distinguish point mismatches becomes more robust.

Furthermore, rapid and robust SNV detection assays also play an important role in the fields of food safety and industrial biotechnology, where they can be used for quality controls on microbes producing different microbial products such as antibiotics, antibodies, products obtained through fermentation, and metabolites, etc, Fig. 4e (Dincer et al., 2017; Yao et al., 2018; Tang and Fu, 2018; English et al., 2019; Williams et al., 2019; Gulati et al., 2021; Pan et al., 2022; Zhu et al., 2022; Mu et al., 2022; Mao et al., 2022; Qian et al., 2022). Taken together, CRISPR-based single-nucleotide diagnostics have achieved tremendous success in the short period that such modalities have been used for pathogenic SNV detection. Coming years will show how

applicable these are for routine point-of-care surveillance. The cost, affordability, and robustness of the assay would determine the success of these technologies in the long run.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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